REMARKS

As an initial matter, Applicant wishes to thank Examiner Colaianni for the courtesies extended during a personal interview on August 8, 2000 with the inventor, Jonnie Williams, and Applicant's representatives Susan Wolffe and Paul Rivard. Claims 2, 3, 5-9, 11-13, and new claims 53-68 are pending. By the foregoing amendment, non-elected claims 14-19 and 21-50 have been canceled without prejudice. New claim 53 corresponds to original claim 1 and includes the subject matters of dependent claims 4 and 10, which have been canceled. Claims 2, 11, 12, and 13 have been amended to correct their dependencies. New claim 58 replaces claims 51 and 52, which have been canceled. No new matter has been added. Entry of the amendments is respectfully requested.

Claim 53, which replaces canceled claim 1, points out more fully the invention by setting forth that a tobacco plant or portion thereof, while uncured, yellow, and in state susceptible to having the formation of at least one nitrosamine arrested, is dried in a controlled environment and for a time sufficient to substantially prevent the formation of at least one nitrosamine. As described in the specification, *e.g.*, page 10, line 16 to page 11, line 2, the controlled environment can be obtained by selecting an appropriate combination of curing parameters such as airflow, temperature, and humidity. The controlled environment also includes air free of combustion exhaust gases and an airflow sufficient to substantially prevent an anaerobic condition around the vicinity of the plant portion. Anaerobic conditions can result, *e.g.*, from the presence of combustion exhaust gases inside the curing barn or from the release of carbon dioxide by the plant during cure. Such anaerobic conditions are believed to contribute to nitrosamine formation by microbial action (page 14, lines 5-17).

New claim 58, which replaces claims 51 and 52, points out more fully a preferred embodiment of the invention. In claim 58, at least a portion of a tobacco plant, while the portion is uncured, yellow, and in a state susceptible to having formation of said at least one nitrosamine arrested, is heated with convection air for a time sufficient to substantially prevent formation of at least one nitrosamine. The air is free of combustion exhaust gases and substantially prevents an anaerobic condition around the vicinity of said plant.

During the August 8, 2000 interview, Mr. Colaianni questioned whether the limitation "substantially free of combustion exhaust gases" in proposed claims 53 and 58 fully distinguished direct-fired heating because the of the definition of "substantial" in the specification at page 12, lines

17-19. Mr. Colaianni requested that the word "substantially" be deleted from this phrase so as to exclude direct-fired heating. New claims 53 and 58 recite that the air is "free of combustion exhaust gases." It will be understood, of course, that the curing air contains quantities of water vapor and other compounds present in ambient air which may be considered combustion products. Rather, the limitation "free of combustion exhaust gases" is meant only to distinguish direct-fired heating in which combustion gases are readily exhausted into the curing air.

New claims 54-57 and 63-68 recite that, following drying, the content of at least one tobacco-specific nitrosamine, *e.g.*, N'-nitrosonornicotine, 4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone, N'-nitrosoanatabine, and/or N'-nitrosoanabasine, is at least 75%, 90%, or 95% by weight lower than the content of the corresponding tobacco-specific nitrosamine(s) in cured brown tobacco made from the same tobacco crop but which was cured in the absence of steps designed to reduce the content of said at least one nitrosamine. Applicant notes that unlike claims 53 and 58, independent claims 54 and 63 recite that the air is "substantially free of combustion exhaust gases." It is believed that this limitation, particularly taken in combination with the limitations directed to quantitative nitrosamine content in the tobacco, also amply distinguishes direct-fired heating. Claims 67-68 refer to types of tobacco that can be used in the claimed process. Support for new claims 54-57 and 63-68 is found in the specification, *e.g.*, at page 28, lines 4-11, and page 19, lines 19-20.

Restriction Requirement

Applicant confirms its previous telephone election of Group (I), claims 1-13, 51, and 52 to a process of substantially preventing the formation of nitrosamines in a tobacco plant. The election is made without traverse in cooperation with the accelerated examination procedure set forth *inter alia* in M.P.E.P. § 708.02(VIII), the subject application having been granted "special" status by a communication from the USPTO dated April 14, 2000. Non-elected claims 14-19 and 21-50 have been canceled without prejudice herein and will be pursued in a divisional application. Pending claims 2, 3, 5-9, 11-13, and 53-68 all are directed to the elected process.

Claim Rejections Under 35 U.S.C. §§ 102 and 103

Marley U.S. Patent 4,790,335

Claims 1 and 52 stand rejected under 35 U.S.C. § 102(b) as being anticipated by, or in the alternative under 35 U.S.C. § 103(a) as being unpatentable over, Marley U.S. Patent 4,790,335. By the foregoing amendment, claims 1 and 52 have been canceled. New claims 53 and 58 include the limitations of claims 4 and 10. Because claims 4 and 10 were not included in this rejection, the rejection is now moot. Marley is discussed in detail below in the response to the rejection which did include original claims 4 and 10.

Bokelman U.S. Patent 4,355,648

Claim 12 stands rejected under 35 U.S.C. § 102(b) as being anticipated by, or in the alternative under 35 U.S.C. § 103(a) as being unpatentable over, Bokelman U.S. Patent 4,355,648. This rejection is respectfully traversed.

As an initial matter, Applicant notes that claim 12 is a dependent claim which depended on and included all limitations of claim 1. *See* 35 U.S.C. § 112 ¶ 4. It appears from the Office Action that the limitations of claim 1 were not considered in this rejection. New claim 53 replaces claim 1 and also includes the limitations of original claims 4 and 10. Claim 12 now depends on claim 53 and includes all of the limitations of claim 53.

Bokelman describes photobleaching green tobacco followed by thermal browning. *See* column 1, lines 25-28 ("the present invention provides a means for eliminating the green color and green odor and taste of tobacco which is rapid and less labor and energy intensive"). Bokelman does not describe subjecting tobacco to a controlled environment while the tobacco is in a state susceptible to having the formation of nitrosamines arrested, *e.g.*, yellow tobacco, as defined in the subject application and set forth in the process of claim 53.

The Office Action refers to Bokelman as describing tobacco having reduced nitrogen content. However, this statement is made in the context of expressing juices from green tobacco prior to photobleaching (column 1, lines 54-58). Green tobacco is known to contain little or no nitrosamines. Rather, nitrosamines are believed to form primarily during the tobacco curing process. *See, e.g.*, Wiernik *et al.*, "Effect of Air-curing on the Chemical Composition of Tobacco," Recent Advances in Tobacco Science, Vol. 21, pp. 39 *et seq.*, Symposium Proceedings 49th Meeting Tobacco

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Chemists' Research Conference, Sept. 24-27, 1995, Lexington, Kentucky. Bokelman, in fact, states that photobleaching does not drastically alter the chemistry of the tobacco and that tobacco has a form and color similar to conventional flue-cured tobacco (column 2, lines 39-43).

Furthermore, contrary to the Examiner's assertion, nitrogen levels do not correspond to nitrosamine levels. In fact, low nitrogen level tobaccos can end up with high levels of nitrosamines.

Bokelman does not teach or suggest the process of claim 53 and, in fact, simply is irrelevant to the process set forth in claim 53. Thus, Bokelman does not teach or suggest the process of claim 12. Reconsideration and withdrawal of this ground of rejection are respectfully requested.

Buensod U.S. Patent 1,568,316

Claims 1-11, 51, and 52 stand rejected under 35 U.S.C. § 102(b) as being anticipated by, or in the alternative under 35 U.S.C. § 103(a) as being unpatentable over, Buensod U.S. Patent 1,568,316. This rejection is respectfully traversed.

Buensod '316 describes a tobacco curing process for making tobacco leaves used for cigar wrappers (col 1, lines 10-12) "in which conditioned air is caused to be artificially circulated around and between the tobacco leaves in such a schedule of proper temperatures and humidities as to effect in the speediest manner, the desired results of cell starvation, etiolation of green color, control of the brown coloration and the incidental oxidation while at the same time preserving the cell structures thereby developing the elasticity of the tobacco" (column 1, lines 16-25). The Office Action concedes Buensod '316 does not describe or discuss in any way nitrosamine formation or content in tobacco.

In the process disclosed by Buensod '316, green leaves are placed in a barn and heated with re-circulated air at a temperature of 95°F to 110°F. The cells of the tobacco are said to be heated to a temperature above that of the wet bulb and below that of the dry bulb temperature of the air. This condition is said to be quickly reached and then humidity is regulated. Relative humidity is maintained at 70-75% for about 24 hours as the process of respiration and etiolation continues (page 8, lines 71-78).

After 24 hours, relative humidity then is increased and maintained at 75-80% at the same temperature or at a gradually decreasing temperature during the remainder of the curing phase (page 8, lines 79-99). Buensod's curing conditions of relatively high humidity levels in combination with

moderate curing temperatures, particularly when the tobacco is in what the present application defines as a susceptible state, are extremely conducive to nitrosamine formation. Buensod '316 simply does not describe a controlled environment capable of substantially preventing the formation of nitrosamines as defined and claimed in the subject application.

Burton *et al.*, *Changes in Chemical Composition of Burley Tobacco during Senescence and Curing. 3. Tobacco Specific Nitrosamines*, J. Agric. and Food Chem., pp. 426-430 (1989) (copy attached), discusses the formation of nitrosamines in Burley tobacco during curing. Table II therein reports nitrosamine levels (NNN, NAT, and NNK) measured in Burley tobacco at various stages of curing carried out at 32°C (about 90°F) and at about 83% relative humidity. These conditions are representative of the curing conditions described by Buensod '316, as discussed above. Burton reports that such conditions actually yielded tobacco having higher nitrosamine levels compared to conventionally air-cured tobacco (page 429). The results reported by Burton are equally applicable to other types of tobacco.

The Burton publication evidences that the conditions described in Buensod '316 would not inherently or obviously prevent the formation of nitrosamines as set forth in the claimed invention. Nothing in Buensod describes or suggests a process which would substantially prevent the formation of nitrosamines in tobacco. Indeed, Buensod '316 describes curing conditions which are particularly conducive to nitrosamine formation. Buensod '316, if anything, leads one skilled in the art away from the claimed invention.

It is further noted that Buensod '316 is looking for a tobacco having a particular color or aroma. These criteria can not be used to determine nitrosamine levels. In fact, a "perfect aroma" would likely have higher levels of nitrosamines due to the conditions required to obtain such an aroma. Reconsideration and withdrawal of this ground of rejection are respectfully requested.

Marley and optionally in view of Wilson U.S. Patent 3,664,034

Claims 2-11 and 51 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Marley and optionally in view of Wilson U.S. Patent 3,664,034. This rejection is respectfully traversed.

Marley describes an apparatus for curing tobacco which employs a gas tank (typically propane) for fueling dual heating systems which heat separate chambers (52, 54) for curing the

leaves and stems, respectively. Combustion exhaust gases produced by burning the gas fuel source in heater blowers 40 and 42 are circulated through the open conveyor chambers during curing of the tobacco to somehow provide zones with controlled temperatures.

The description in the specification along with the apparatus shown in the drawings depict direct-fired heating. Direct-fired heating was a standard in the industry at the time the Marley application was filed. Since Marley describes the necessary elements to achieve direct-fired heating, there is no reason to believe that Marley heats by any other means.

Further, there is no provision for exhausting combustion gases except through the lines leading to the heating chambers. Thus, Marley does not describe taking any steps to remove combustion exhaust gases from the heated air. Moreover, there is no reason that one skilled in the art would have removed combustion exhaust gases based on the Marley disclosure. Marley certainly does not recognize that combustion gases can be detrimental to the curing process of tobacco. Specifically, Marley does not recognize in any way that combustion gases can promote the formation of nitrosamines in tobacco.

In order to simulate the procedure described by Marley, a modified Sharp Carousel microwave convection oven was used to treat tobacco under the conditions described in Marley. See the attached copies of the Declarations of Harold R. Burton, Ph.D. and Paul H. Lamb, III, which originally were submitted in parent application Serial No. 08/998,043. A propane flame was applied intermittently through an inlet pipe of the converted convection oven to maintain the desired temperatures. The air circulation feature of the oven was retained and air was recirculated as well as exhausted to the outside through vents in the rear of the oven.

As reported in the Declarations, the Marley arrangement was found to be ineffective. Rapid heating of the tobacco according to the Marley directions, as expected, was ineffective to achieve a cure. Rather, the green color was locked in the tobacco. Therefore, Marley does not describe treating tobacco while it is in a susceptible state, in particular yellow tobacco, as defined and claimed in the subject application.

Moreover, Marley does not describe or suggest treating tobacco under any conditions which would substantially prevent nitrosamine formation as is now claimed. The Office Action's contention that the conditions described in Marley are the same as those of the present invention and inherently or obviously would result in the same levels of nitrosamine formation, is amply refuted

by the tests reported in the attached declarations. Marley does not discuss nitrosamine content and provides no guidance whatsoever as to how nitrosamine formation can be prevented.

Marley also does not describe air which is free or substantially free of combustion exhaust gases as set forth in independent claims 53, 54, 58, and 62. Indeed, the Marley arrangement is direct-fired and does nothing to prevent combustion exhaust gases from entering the curing chamber during drying of the tobacco. The presence of combustion exhaust gases within tobacco curing chambers is known to create anaerobic conditions which contribute to the formation of nitrosamines in tobacco through microbial action. *See* Burton Declaration, ¶ 16.

Paragraph 12 of the Office Action incorrectly states that the heater/blower is not disclosed as being gas or oil-fired. As discussed above, a gas tank (28) fuels the heater/blower in the Marley arrangement (col. 3, lines 4-11), and no provision is made for preventing combustion exhaust gases from entering the curing chamber (See Fig. 2). This is referred to in the art as "direct-fired" heating. The air inside Marley's curing chamber is not free of combustion exhaust gases as the Office Action contends.

Thus, Marley does not teach or suggest the process of the present claims. Wilson does not remedy the defects of Marley. Wilson is directed to a system of curing tobacco by controlling the temperature, amount of air being circulated, and relative humidity. However, Wilson does not teach or suggest curing tobacco under conditions that would substantially prevent the formation of nitrosamines. In addition, although Marley mentions using a heat exchanger system with an oil-fired system, the specific curing system described by Wilson is gas-fired, similar to the system used by Marley. In such a gas-fired system, the combustion gases are introduced directly into the combustion chamber. This arrangement is preferable with gas-fired systems. See column 3, lines 62-65. Thus, one skilled in the art would not have been motivated to remove the combustion gases from the direct gas-fired system of Marley based on Wilson.

In particular, Wilson discloses a barn having air flow throttling damper members for selectively adjusting air flow during yellowing and drying (column 5, lines 30-32 and column 6, lines 53-71). As stated above, Although Wilson mentions the use of a heat exchanger for oil-fired systems, Wilson specifically describes a gas-fired burner (46) which communicates directly with the interior of the housing, *i.e.*, products of combustion pass into the curing air (column 3, lines 62-65). A fresh air inlet also has a damper member to adjust the amount of air introduced to control relative

humidity of the curing air (column 3, line 74 to column 4, line 15). The gas-fired system of Wilson does not provide air free of combustion exhaust gases in accordance with the present claims. Since this is the preferred system, clearly Wilson does not appreciate the impact of combustion gases in the formation of nitrosamines.

Wilson does not remedy the deficiencies of Marley and does not describe or lead one skilled in the art to the claimed invention. Reconsideration and withdrawal of this ground of rejection are respectfully requested.

Marley in view of Hopkins U.S. Patent 4,430,806 or Wochnowski U.S. Patent 4,898,189

Claim 13 stands rejected under 35 U.S.C. § 103(a) as being unpatentable over Marley in view of Hopkins U.S. Patent 4,430,806 or Wochnowski U.S. Patent 4,898,189. This rejection is respectfully traversed.

Hopkins and Wochnowski describe the use of microwave energy for driving off moisture from agricultural products such as uncured, green tobacco. Neither document describes applying microwave energy to tobacco while the tobacco is in a susceptible state so as to substantially prevent formation of at least nitrosamine. Both Hopkins and Wochnowski were considered (and distinguished) during prosecution of U.S. Patent 5,803,081 to the present inventor, directed to a method of reducing nitrosamine levels or preventing formation of nitrosamines in a harvested tobacco by exposing tobacco, while it is uncured and in a susceptible state, to microwave energy.

Neither Hopkins nor Wochnowski describes a controlled environment capable of substantially preventing the formation of nitrosamines in a tobacco plant as set forth in claim 53. Therefore, neither Hopkins nor Wochnowski remedies the deficiencies of Marley as discussed above. Claim 13 distinguishes the cited references at least for the same reasons that claim 53 distinguishes the references. Reconsideration and withdrawal of this ground of rejection are respectfully requested.

CONCLUSION

In view of the foregoing, favorable reconsideration of the subject application is respectfully requested.

Respectfully submitted,

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Date: August 9, 2000

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Changes in Chemical Composition of Burley Tobacco during Senescence and Curing. 3. Tobacco-Specific Nitrosamines

Harold R. Burton,* George H. Childs, Jr., Roger A. Andersen, and Pierce D. Fleming

Burley tobacco was harvested at three stages of maturity and cured in two curing environments (24 °C/70% RH, 32 °C/83% RH). Tobacco samples taken throughout the curing process were analyzed for *N'*-nitrosonornicotine (NNN), *N'*-nitrosoanatabine (NAT), 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK), nitrate nitrogen, and nitrite nitrogen. There was a positive correlation between nitrite nitrogen and nitrosamines when cured in a normal environment (24 °C/70% RH). Curing tobacco at a higher temperature and humidity (32 °C/83% RH) dramatically increased the accumulation of individual nitrosamines and nitrite. Again, a significant, positive correlation was observed between tobacco-specific nitrosamines (TSNA) and nitrite.

The detection of tobacco-specific nitrosamines (TSNA) in cured tobacco led to several studies to identify the precursors of these nitrosamines and conditions required for their accumulation. Brunnemann et al. (1983) indicated that a positive correlation existed between nitrate content of the leaf and TSNA. Studies by Djordjevic et al. (1985, 1987) and MacKown (1988) indicated that a positive correlation existed between *N'*-nitrosonornicotine (NNN) and nornicotine and *N'*-nitrosoanatabine (NAT) and anatabine. Parsons et al. (1986) indicated that on the homogenized leaf curing of burley tobacco (HLC) there was reduction of nitrate to nitrite. In an independent study on burley tobacco Andersen and Kemp (1985) showed that the increase of nitrite during HLC was accompanied by an increase of NNN. It was shown that TSNA increases during air-curing of burley tobacco (Djordjevic et al., 1985, 1987; Andersen et al., 1987); however, it is not known whether there is a specific time during curing when TSNA accumulation is optimal. Therefore, this study was initiated to determine if plant maturity and/or curing environment influenced the accumulation of TSNA and nitrite and when TSNA and nitrite accumulation occurred during curing.

EXPERIMENTAL SECTION

Nicotiana tabacum L. cv. Ky 14 was grown at the Kentucky Agricultural Experiment Station farm in 1985 with standard agronomic practices for burley tobacco production. Tobacco was harvested 1, 4, and 7 weeks after topping. The tobacco plants were cut and placed on sticks (6 plants/stick) and cured (18 sticks, 108 plants/chamber) in two controlled environmental chambers. The chambers were maintained at 24 °C/70% RH and 32 °C/83% RH, respectively. These conditions effected identical moisture losses from the tobacco lamina during curing (Walton et al., 1982). Three replicate samples (3 leaves/plant and 3 plants/replicate) were taken at 0, 1, 2, 3, 5, 7, 9, 12, 14, 16, 19, and 21 days after harvest from the top one-third of the plant. It should be noted each plant was sampled once. The samples were separated into lamina and midvein, weighed, freeze-dried, reweighed, ground to pass a 40-mesh screen, and stored at −40 °C until analyses.

Chemical Analyses. Nitrate was determined by *Escherichia coli* reduction as described by Lowe and Gillespie (1975). Nitrite was determined by the same procedure that was similar to that for nitrate except the *E. coli* was omitted and the procedure was modified as follows. One-gram samples were weighed into 25 × 200 mm screw-cap test tubes and extracted by shaking with 25 mL of water for 1 h on a reciprocal shaker. Samples were then filtered through Whatman No. 1 filter paper and a 10-mL aliquot was decolorized with 1.0 g of Norit A activated charcoal, by reciprocal shaking for 10 min. The samples were then filtered through Whatman No. 42 filter paper and analyzed colorimetrically for nitrite on a Technicon autoanalyzer system II. The manifold assembly described by Lowe and Gillespie (1975) was modified to achieve greater sensitivity by increasing the sample tube size (0.42 mL/min), decreasing the diluent tube size (0.60 mL/min), and substituting a 50-mm flow cell in the colorimeter. Also the delay coil in the heating bath was bypassed to decrease the system retention time.

Calcium was quantified by atomic absorption spectrometry of 8:1 perchloric acid–nitric acid digests of the tobacco samples.

Analyses of Tobacco-Specific Nitrosamines. *N'*-Nitrosonornicotine (NNN), *N'*-nitrosoanatabine (NAT), and 4-(*N*-methyl-*N*-nitrosoamino)-1-(3-pyridyl)-1-butanone (NNK) were analyzed by capillary GC using a nitrogen-specific detector as previously described (Andersen and Kemp, 1985). Their identities were confirmed by GC–MS using a Finnigan 700 ion trap detector interfaced with a Varian 3700 GC equipped with an on-column injector. Recovery–response factors were determined for each authentic nitrosamine, and azobenzene was used as the internal standard.

RESULTS AND DISCUSSION

Influence of Plant Maturity and 24 °C/70% RH Curing Conditions on Accumulation of TSNA. Data for the accumulation of tobacco-specific nitrosamines (TSNA) during air-curing at 24 °C/70% RH are presented in Table I. Results from three harvest dates are presented in order to show that plant maturity influenced the accumulation of nitrosamines. Since these tobaccos were cured in an environmentally controlled curing chamber, differences in nitrosamine accumulation should be due to plant maturity and not due to differences of the curing environments.

Immature tobacco, harvested 1 week after topping, contained low concentrations of NNN (Table I): however, there was a 5-fold increase in NNN between harvest and final sampling (21 days). The most significant increase of NNN occurred between the 14th and 16th day after harvest. This increase occurred at the end of yellowing,

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Table I. Changes of Tobacco-Specific Nitrosamines, Nitrate, and Nitrite during Air-Curing (24 °C/70% RH) of Burley Tobacco Harvested at Three Stages of Maturity

day	NNN/μg/g	NAT/μg/g	NNK/μg/g	total/μg/g	NO_3 N/mg/g	NO_2 N/μg/g
			Harvest 1[a]			
0	0.40	1.69	0.80	2.16	4.33	0.64
1	1.33	1.37	1.17	3.87	6.14	0.72
2	0.83	1.17	0.07	2.07	5.73	0.63
3	0.72	0.48	0.08	1.28	6.19	0.43
5	0.54	6.34	0.18	7.06	5.93	0.60
7	0.94	1.61	0.16	2.71	6.78	0.54
9	0.54	0.69	0.06	1.29	6.34	0.37
12	0.75	2.40	0.40	3.55	7.01	0.86
14	0.65	5.79	0.13	6.58	7.29	0.43
16	1.99	4.56	0.37	6.92	8.43	1.11
19	1.32	3.35	0.32	4.99	9.21	2.35
21	2.06	3.31	0.47	5.84	8.61	7.38
LSD (05)	0.40	1.19	0.18	1.84	1.36	1.92
			Harvest 2[b]			
0	0.43	1.98	0.13	2.54	4.42	0.62
1	1.40	1.77	0.10	3.27	3.13	0.73
2	1.12	0.86	0.20	2.18	4.26	0.55
3	1.62	1.17		2.79	4.23	0.38
5	1.58	3.65	0.20	5.44	3.78	2.50
7	2.10	4.98	0.45	7.53	4.39	0.83
9	2.17	3.50	0.25	5.92	4.61	1.05
12	2.63	8.76	0.91	12.30	3.87	3.49
14	3.21	8.39	0.49	12.09	5.21	3.42
16	2.25	3.55	0.13	5.93	3.29	1.68
19	5.45	9.82	0.54	15.81	4.12	5.08
21	3.51	5.63	0.17	9.31	3.99	1.39
LSD (05)	2.05	2.79	0.43	4.30	0.86	1.49
			Harvest 3[c]			
0	0.56	2.14	0.10	2.80	1.15	0.47
1	3.03	4.35	0.50	7.88	2.67	2.16
2	2.39	3.67	0.21	6.27	2.96	0.74
3	2.75	3.48	0.13	2.88	3.61	0.71
5	2.80	4.18	0.42	7.40	3.81	1.53
7	2.58	3.71	0.62	6.73	4.54	1.19
9	1.91	2.28	0.37	4.56	4.14	1.11
12	2.89	3.29	0.49	6.66	3.71	1.47
14	4.74	4.31	0.61	9.66	4.03	2.63
16	4.64	6.83	0.31	11.79	4.58	3.85
19	3.79	7.00	0.54	11.33	3.74	4.73
21	3.38	5.69	0.41	9.47	4.26	2.43
LSD (05)	2.45	4.15	0.46	6.38	1.19	3.17

[a] Harvested 1 week after topping. [b] Harvested 4 weeks after topping. [c] Harvested 7 weeks after topping.

an event corresponding to the time when 98% of the chlorophyll was degraded in lamina and lamina leaf color had changed from yellow to brown (Burton et al., 1983, 1985; Tso, 1972). For tobacco harvested at 4 weeks after topping (harvest 2, recommended harvest time), the increase of NNN was significant by the 12th day after harvest. There was a gradual increase of NNN from harvest until the 9th day; however, this increase was not statistically significant. Again, the higher levels of NNN that accumulated by the 14th day occurred after yellowing and during browning of the lamina.

There was a significant increase of NNN 1 day after harvest for the overmature tobacco (harvest 3). Concentrations of NNN remained high throughout the air-curing process, indicating the accumulation of NNN occurred at earlier stages of curing in comparison to the other harvests. The level of NNN increased during the latter stages of curing, but when adjusted for dry-matter loss (Burton and Crutchfield, 1988), there was no increase in the concentration of the TSNA. Results from this study showed that plant maturity influenced accumulation of *N'*-nitrosonornicotine and the increase in NNN occurred within 1 day after harvest for the overmature tobacco. This latter observation would indicate that a significant increase occurred in the tobacco before desiccation of the lamina (Burton and Kasperbauer, 1985) (Figure 1).

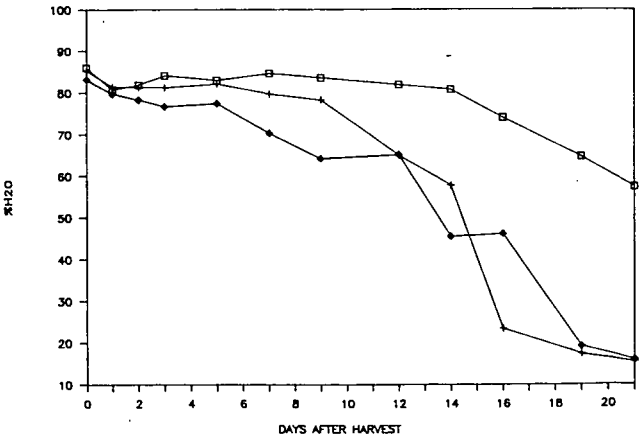

Figure 1. Moisture content in lamina during curing at 24 °C/70% RH: □, harvested 1 week after topping; +, harvested 4 weeks after topping; ◇, harvested 7 weeks after topping.

The accumulation of *N'*-nitrosoanatabine (NAT) during air-curing paralleled the accumulation of NNN. This would be predicted since anatabine, the probable precursor for NAT, is present in these tobacco samples. However, for almost every sampling date, NAT concentration was greater than the NNN level. This is of interest since initial

Table II. Changes of Tobacco-Specific Nitrosamines, Nitrate, and Nitrite during Air-Curing (32 °C/83% RH) of Burley Tobacco Harvested at Three Stages of Maturity

day	NNN/μg/g	NAT/μg/g	NNK/μg/g	total/μg/g	NO_3 N/mg/g	NO_2 N/mg/g
			Harvest 1[a]			
0	0.40	1.69	0.08	2.16	4.33	<0.01
1	1.33	1.37	1.17	3.87	6.14	<0.01
7	0.33	12.72	0.14	13.19	6.71	0.02
12	1.72	3.13	0.77	5.63	6.07	0.03
14	4.09	18.48	1.77	24.33	7.01	0.03
16	12.19	36.65	3.85	52.69	7.77	0.05
19	54.17	333.18	115.69	503.04	7.90	0.93
21	88.33	298.48	118.90	505.72	8.18	1.00
LSD (0.5)	51.94	123.96	61.38	345.94	1.44	0.37
			Harvest 2[b]			
0	0.43	1.98	0.13	2.54	4.42	<0.01
1	1.40	1.77	0.10	3.27	3.13	<0.01
7	4.79	34.30	3.78	42.88	4.71	0.03
12	3.52	18.60	2.70	24.81	4.50	0.05
14	22.40	66.33	11.91	100.64	5.55	0.11
16	30.98	100.75	21.33	153.06	5.98	0.63
19	221.96	545.49	145.46	912.91	5.03	1.00
21	21.57	153.48	28.77	203.81	3.96	0.51
LSD (05)	70.58	79.60	24.78	105.74	1.38	0.35
			Harvest 3[c]			
0	0.56	2.14	0.10	2.80	1.15	<0.01
1	3.03	4.35	0.50	7.88	2.67	<0.01
7	2.94	20.32	0.90	24.16	3.97	0.02
12	32.28	90.75	1.01	133.77	4.63	0.03
14	12.21	32.83	4.40	49.44	4.98	0.07
16	41.75	78.09	6.33	133.58	5.88	0.36
19	20.41	95.52	10.79	126.71	5.93	0.29
21	71.04	145.74	18.24	235.02	7.25	0.25
LSD (05)	42.43	63.57	11.86	107.88	1.41	0.18

[a] Harvested 1 week after topping. [b] Harvested 4 weeks after topping. [c] Harvested 7 weeks after topping.

results indicated that there was the same specificity for the accumulation of NAT as there was NNN. Plant maturity also influenced the accumulation of this nitrosamine. The immature cured tobacco contained the lowest level of NAT whereas the mature (4 weeks) and overmature (7 weeks) cured tobacco contained the highest levels of NAT. For tobacco harvested 4 weeks after topping, there was a significant increase of NAT between days 9 and 12 after harvest. This increase corresponded to the moisture in the lamina (Figure 1). This moisture loss suggested loss of cell integrity (Burton et al., 1983), and loss of cell integrity would allow for invasion of exogenous microbes into the dying tobacco cells. The microbes may be indirectly responsible for the formation of the nitrosamines, since some of the identified microbes on tobacco are nitrate-reducing organisms (Parsons et al., 1986; Douglass et al., 1978).

When tobacco was harvested at 7 weeks after topping, NAT increased 1 day after harvest but the increase was not significant until 16 days after harvest, in comparison to the harvest date.

The mean values for NNK throughout curing, except for one case, were less than 1.0 μg/g. Even though there were some statistically significant values, there were no trends between the NNK content and the different stages of curing. It was evident that, under controlled curing at 24 °C/70% RH, there was no significant increase in the concentration of this biologically active nitrosamine.

Summation of the concentration of each individual tobacco-specific nitrosamine (NNN, NAT, NNK) reflected the net accumulation of these nitrosamines during curing. Over 90% of the TSNA accumulation was due to increases of the NNN and NAT content during the curing process (Table I). For the first two harvests, increases occurred during the third week of curing. TSNA was also highest during the third week of curing in the overmature tobacco (harvest 3). Using a general linear model for comparing data from these three harvests (data not shown) indicates that NNN, NAT, and total are significantly lower for the immature tobacco. This shows that time of harvest influenced the concentration of nitrosamines that accumulated in the lamina.

Data from these curing studies showed there was an increase of TSNA during curing, but there is no explanation for these observed increases. It was assumed that as nitrosamines increase, there also should be an increase of nitrite levels in the lamina during the curing process. This is based on the premise that nitrite is required for nitrosamine formation (Mirvish, 1975; Douglass et al., 1978). Because of low concentrations of nitrite in air-cured burley tobacco (<10 μg/g), an analytical procedure was developed to determine nitrite concentration between 0.040 and 10 μg/g (Burton and Crutchfield, 1988). By using this procedure, it was possible to determine more precisely the concentration of low levels of nitrite in tobacco from harvest through air-curing.

Values for nitrite concentration are presented in Table I along with the nitrate concentrations. Nitrate values were included to show that they were not correlated with nitrite concentrations or the tobacco-specific nitrosamines. The mean nitrite concentrations from all harvest dates range from 0.4 to 7.38 μg/g, which is an over 18-fold difference in nitrite concentration in the tobacco lamina. For all three harvests, the nitrite concentrations were highest during the third week of curing. Generally when there were higher concentrations of nitrite, there were correspondingly higher concentrations of the nitrosamines. Correlation coefficients between nitrite and the individual nitrosamines in Table I show that there were significant and positive correlations between nitrite and all individual nitrosamines at the 99% confidence level. Correlation coefficients between nitrite and NNN, NAT, NNK, and total TSNA were 0.60, 0.56, 0.44, and 0.49, respectively.

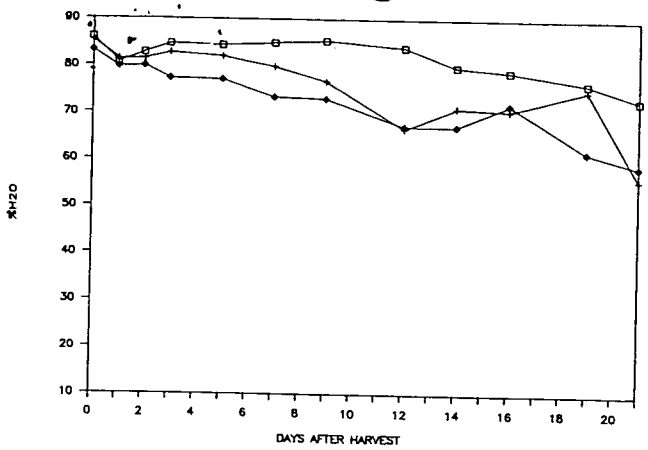

Figure 2. Moisture content in lamina during curing at 32 °C/83% RH: □, harvested 1 week after topping; +, harvested 4 weeks after topping; ◊, harvested 7 weeks after topping.

These data showed that, under an environment considered ideal for curing burley tobacco, nitrite concentration was important for the accumulation of tobacco-specific nitrosamines. It should be noted again there was no significant correlation between nitrate and the individual as total nitrosamine levels.

Influence of Plant Maturity and 32 °C/83% RH Curing Conditions on Accumulation of TSNA. Another aspect of this study was to determine whether increased temperature and relative humidity influenced the accumulation of tobacco-specific nitrosamines and nitrite. Results for TSNA, nitrite, and nitrate from burley tobacco harvested at three stages of maturity and cured at 32 °C/83% RH are presented in Table II. Individual TSNAs were low at harvest and increased significantly during the later stages of curing. During curing of tobacco at the higher temperatures and relative humidities, the total TSNA concentration approached 1 mg/g. This was approximately a 400-fold increase of the nitrosamine concentration during the curing process. Even though these high TSNA values did not occur for conventionally air-cured burley tobacco, these data indicated that if higher temperature and relative humidities were maintained during the latter stages of curing, the resulting tobacco could contain significantly higher levels of nitrosamines. Multivariate analyses of the data in Table I and II showed (data not presented) there is a statistically significant influence of temperature/relative humidity on the accumulation of all individual nitrosamines.

The increase of nitrosamines in tobacco was most likely due to the high levels of nitrite that accumulated during the curing process. For the first and second harvests, there was 100-fold increase of nitrite concentration. This increase of nitrite occurred between the second and third weeks after harvest for all three harvests and corresponded to the increases of the individual nitrosamines and total TSNAs. Correlations between nitrite and NNN, NAT, NNK, and total TSNA were 0.77, 0.91, 0.93, and 0.90, respectively. These correlations were significant at the 99.9% confidence level. It should be noted there was no significant correlation between nitrate concentrations and the individual nitrosamines or the total tobacco-specific nitrosamines. The absence of correlation between nitrate and nitrite would be expected since the nitrate concentration was constant except for changes that occurred during the curing process.

Accumulation of high levels of nitrite during the latter stages of curing at the higher temperature and relative humidity may be due to enhanced activity of exogenous nitrite-reducing organisms. Even though the color of tobacco lamina changed from green to yellow to brown, indicating a normal curing process, the moisture content only decreased to 56% (Figure 2). After 21 days, this high moisture content in the cured lamina should enhance microbial activity in the cured lamina. The influence of moisture content in cured lamina on the accumulation of nitrite in cured tobacco lamina is presently being investigated.

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Registry No. NNN, 16543-55-8; NAT, 71267-22-6; NNK, 64091-91-4; NO_3^-, 14797-55-8; NO_2^-, 14797-65-0.

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